Apparent role of traveling metabolic waves in oxidant release by living neutrophils

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Cell metabolism self-organizes into two types of dissipative structures: chemical oscillations and traveling metabolic waves. In the present study we test the hypothesis that traveling NAD(P)H waves within neutrophils are associated spatially and temporally with the release of reactive oxygen metabolites (ROMs). Using high-speed optical microscopy and taking advantage of the autofluorescence of NAD(P)H, we have observed the propagation of NAD(P)H waves within cells. When NAD(P)H waves reach the lamellipodium of morphologically polarized neutrophils, a diffusing plume of superoxide is released as evidenced by the conversion of hydroethidine in the extracellular environment to ethidium bromide. Parallel results were obtained by using high-speed emission microspectrophotometry. These experiments indicate that the spatial and temporal properties of NAD(P)H waves are transformed into ROM pulses in the extracellular environment. Propagating NAD(P)H waves allow neutrophils to specifically deliver substrate to the lamellipodium at high concentrations, thus facilitating the local and periodic release of ROMs in the direction of cell movement and/or a target.

Thermodynamic principles account for the self-organization of biological molecules such as protein folding, lipid bilayer assembly, and the winding of a DNA helix. In addition to these seemingly equilibrium phenomena driven by entropy, nonequilibrium processes also may contribute to cell structure and function. Living cells are constrained far from equilibrium by a constant flux of matter and energy (1). Nonequilibrium conditions permit the formation of chemical oscillations and waves, which are called “dissipative structures” because they dissipate some of the energy absorbed from the environment to support their self-organization (2). One well known oscillator exhibiting temporal oscillations and spatiotemporal patterns is glycolysis (3). Although formation of dissipative metabolic structures had been predicted (4–7) and observed in cell extracts (8–11), it has been only recently possible to detect these structures in living cells (12–14). However, it has not been established whether dissipative metabolic structures represent a physical curiosity in cells or an important biological regulatory mechanism. Using high-speed microscopic and microspectrophotometry, we show that intracellular traveling NAD(P)H waves temporally and spatially correlate with oxidant release plumes from neutrophils. Our findings support a biological role of dissipative structures in intracellular communication and provide insight into the physical-chemical mechanisms underlying inflammatory responses at the cellular level.

Neutrophils are key participants in inflammatory responses such as autoimmune disease, ischemia-reperfusion damage (e.g., myocardial infarction and transplantation), and host resistance to cancer and infection. One mechanism mediating target destruction is the production of superoxide anions and their downstream reactive oxygen metabolites (ROMs) including H₂O₂, O₂⁻, and HOCl. Receptor-mediated signaling for cell activation increases the glucose influx rate (15). Glucose uptake, which drives the system further away from equilibrium, is required for ROM production (16). Alterations in glucose influx have been predicted to change metabolic oscillations (17), which is consistent with data from this laboratory (ref. 14; unpublished data). Transmembrane signaling also leads to phosphorylation of the NADPH oxidase, which is necessary for heightened ROM production (e.g., ref. 18). Superoxide is produced according to the relationship

$$2O_2 + NADPH \rightarrow 2O_2^- + NADP^+ + H^+.$$ [1]

It is well established that superoxide production, as well as other neutrophil properties, oscillate (19), although their mechanistic origin(s) has not been established firmly. We have proposed that ROM oscillations are a consequence of oscillating NADPH levels (19). One implication of this concept is that intracellular traveling NAD(P)H waves (12–14) set the spatial and temporal coordinates of ROM deposition, which is tested in the present study. Neutrophils are a superb model system to test the physical constraints on cell activation because they (i) rely largely on glycolysis for energy production (20), (ii) spread on surfaces, thereby thinning their cytoplasm, and (iii) produce large amounts of ROMs, especially when adherent to a substrate (21).

Materials and Methods

Materials. Hydroethidine (HE), N-formyl-methionyl-leucyl-phenylalanine (FMLP), N-tert-BOC-Phe-Leu-Phe-Leu-Phe, and superoxide dismutase (SOD) from bovine erythrocytes were obtained from Sigma. Dihydrotetramethylrosamine (H₂TMRos) was obtained from Molecular Probes.

Cells. Human peripheral blood neutrophils were purified as described previously by using step-density gradient centrifugation on Histopaque 1077 and 1119 solutions (Sigma; ref. 14). The cells were >95% viable as judged by trypan blue exclusion. The value of n for each of the experiments listed below represents the number of different days on which the experiments were repeated.

Imaging Spectrophotometry. Cells were observed microscopically at 37°C by using quartz slides and quartz coverslips. High-speed image acquisition was performed by using an axiovert fluorescence microscope with a quartz condenser, quartz objectives, and an AttoArc HBO 100-W mercury lamp (Zeiss). To excite simultaneously NAD(P)H autofluorescence and ethidium bromide (EB) fluorescence, cells were illuminated by using a 365WB50 excitation filter, and fluorescence was collected by using a 400-nm long-pass dichroic mirror and a 450AF55 emission filter (Omega Optical, Brattleboro, VT). To increase light-collection efficiency, the microscope’s bottom port was used. This port was fiberoptically coupled to the input side of an Acton-150 (Acton Instruments, Acton, MA) imaging spectrophotometer. The exit side was connected to an intensifier...

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Abbreviations: ROM, reactive oxygen metabolite; HE, hydroethidine; FMLP, N-formyl-methionyl-leucyl-phenylalanine; SOD, superoxide dismutase; H₂TMRos, dihydrotetramethylrosamine; EB, ethidium bromide.

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Gigabyte of random-access memory (RAM), 16 megabytes of TX) Precision 410 workstation with an 800-MHz processor, 1.0 Princeton ST-133 interface and a Stanford Research (Sunnyvale, Trenton, NJ). The camera was controlled by a high-speed high-speed imaging apparatus described above only accommodated several seconds of recording depending on the delay time between frames. To improve the likelihood of capturing high-contrast images of oxidant release, which is especially important at nanosecond shutter speeds, cells were observed by using the live spectrum-analysis mode of the WINSPEC software. Having established a rhythm in the spectroscopy mode, we were able to approximate the timing of the EB fluorescence burst to manually trigger high-speed imaging. Nonetheless, we were successful only in catching the high-intensity burst from beginning to end in about one-half of the cells observed. The instability of the operating system also occasionally caused data losses.

Microspectrophotometry experiments were performed with the apparatus described above. However, in this case the mirror in the Acton unit was replaced with a ruled grating (300 grooves/mm; ref. 12). In these experiments H2TMRos was added to the extracellular environment. Because NAD(P)H and TMRos are excited by different wavelengths of light, a multipass dichroic mirror and filter set was necessary. The XF-59 set from Omega Optical was used.

Results

Spontaneously Polarized Cells. When substrate-associated neutrophils become morphologically polarized (exhibiting a well-defined leading edge and trailing uropod), they display sustained spontaneous polarization of these NAD(P)H waves, as shown in Figs. 1 and 2. The extracellular plume of EB fluorescence is observed immediately after NAD(P)H reaches the lamellipodium. In addition to this temporal coherence, spatial coherence is observed also, because the wave is propagating toward the lamellipodium. The time delay between NAD(P)H arrival and EB formation (≈10 msec) likely is accounted for by electron trafficking across the membrane, diffusion and reaction with oxygen, diffusion of superoxide, and the conversion of HE to EB. We propose that the neutrophil’s self-organized NAD(P)H waves lead to a periodic release of superoxide and that the location of superoxide release is associated with the direction of wave motion.

Although Figs. 1 and 2 clearly indicate a diffusive plume, we next sought to analyze its properties more carefully. To do this, data were acquired by using a 0.1-μsec exposure with a 5–8-msec delay between frames. The oxidative plume was characterized by line-profile analysis with a region extending colinear with cell orientation from the lamellipodium for a distance of 15 μm. To reduce noise, the width of the line was one μm. Fig. 3 shows the results of a typical experiment. The time-dependent relaxation of the EB concentration is evident. The result shown on the far left side of the figure is caused by NAD(P)H arrival at the lamellipodium. To the right of the dashed line of Fig. 3 is the extracellular environment. Although these data are composed of several chemical steps, including Eq. 1 and the conversion of HE to EB, one can estimate a diffusion coefficient of $≈2 \times 10^{-5}$ cm$^2$/sec from these data (25), which is consistent with that expected for a small fluorescent molecule (26).

To confirm and further develop this concept, high-speed spectrophotometry experiments were performed by using extracellular H$_2$TMRos (1 μg/ml) to detect ROM (i.e., H$_2$O$_2$, OH$^-$) release. To allow simultaneous detection of NAD(P)H and the fluorescence-reaction product TMRos, multispot excitation and emission filters and a multipass dichroic mirror were used (Fig. 4). By observing two emission regions using spectrophotometry, we simultaneously monitored NAD(P)H and TMRos emission over time. Because NAD(P)H waves travel throughout a cell, we analyzed only data from a portion of the cytoplasm and extracellular environment on either side of the lamellipodium.
Fig. 1. Representative fluorescence images of polarized human neutrophils at 37°C using high-speed microscopy. HE was added to the medium to detect superoxide release from cells. Optical filters allowed simultaneous observation of NAD(P)H autofluorescence and EB emission. Each image was collected for 100 nsec with a 25-msec interval between frames. For clarity, the locations of the cells are outlined in the first frames of sequences A–D. (A) A time series of fluorescence micrographs of a neutrophil is shown. Fluorescent stripes propagate from the cell’s uropod to the lamellipodium. Just after NAD(P)H reaches the lamellipodium (frame 7), a plume of fluorescence caused by the oxidation of HE to EB diffuses into the medium (n = 6, where n = number of days on which the findings were reproduced). (B) This experiment was performed in a fashion identical to that of A except that 500 units/ml SOD was added to the extracellular environment to reduce extracellular superoxide levels. Fluorescent plumes are not observed (for example, see frames 5–7), which suggests that superoxide is necessary for the formation of EB plumes (n = 4). (C) FMLP stimulation leads to two propagating NAD(P)H waves. Fluorescent plumes are found for all NAD(P)H waves (frames 4, 5, and 10; arrows). (D) This experiment was performed in a fashion identical to that of C except that 500 units/ml SOD was added to the extracellular environment. Fluorescent plumes are not observed (for example, see frames 3 and 9 for two NAD(P)H waves at the lamellipodium) (n = 4). The background fluorescence is somewhat higher in A and C because of EB formation before acquiring these data from these and other cells on the microscope slide (×980).
A representative series of emission spectra is shown in Fig. 4C. NAD(P)H arrival near the lamellipodium is followed rapidly by oxidation of H₂TMRos, indicating the pericellular presence of ROMs. Thus, high-speed microscopy and spectrophotometry agree that an intermittent wave of NAD(P)H is followed rapidly by a spatially and temporally correlated wave of ROM production.

**Polarized FMLP-Stimulated Cells.** Inasmuch as receptor stimulation enhances ROM production, we activated neutrophils by using 100 nM FMLP, a small molecule resembling bacterial peptides. FMLP is one of several molecules that alter the temporal and spatiotemporal properties of cell metabolism (14, 19). It was added uniformly to the cells, thus promoting chemokinesis. FMLP treatment led to the formation of two NAD(P)H traveling waves (Figs. 1C and 2B), although the receptor antagonist N-tert-BOC-Phe-Leu-Phe-Leu-Phe had no effect (data not shown; see also ref. 14). As expected, SOD inhibited the formation of EB by FMLP-treated neutrophils (Fig. 1D). As each NAD(P)H wave reaches the lamellipodium, a plume of EB fluorescence is observed (Figs. 1C and 2B). However, as noted previously (14), FMLP stimulation triggers the formation of two traveling NAD(P)H waves (14), Spectrophotometry studies us-}

(Fig. 4B). A representative series of emission spectra is shown in Fig. 4C. NAD(P)H arrival near the lamellipodium is followed rapidly by oxidation of H₂TMRos, indicating the pericellular presence of ROMs. Thus, high-speed microscopy and spectrophotometry agree that an intermittent wave of NAD(P)H is followed rapidly by a spatially and temporally correlated wave of ROM production.

**Discussion**

The potential role of self-organized chemical patterns in biology was anticipated by the work of Turing, Prigogine, Hess, and others (8, 27, 28). Although we confirmed the existence of traveling metabolic waves within cells (12–14), their physiological relevance has not been rigorously established. We now show the transformation of intracellular substrate [NAD(P)H] waves to extracellular ROM product waves by high-speed microscopic imaging and microspectrophotometry. These experiments show that the site of ROM release corresponds to
the location of NAD(P)H waves and that ROM release immediately follows NAD(P)H arrival at the lamellipodium. The local NADPH concentration apparently switches the NADPH oxidase, a protein found in the leukocyte membrane (29), between “on” and “off” states in cells; hence, enzymes can decode self-organized chemical patterns to yield spatiotemporal patterns of cell activity. Thus, we believe that the physiological importance of intracellular metabolic waves has been established.

Neutrophil activation is a crucial event in many disease processes including host resistance to infectious agents, sepsis, ischemia-reperfusion injury (myocardial infarction, transplantation, etc.), resistance to cancer, and autoimmune disease (e.g., arthritis). When adherent, polarized cells are activated by FMLP, the dissipative structures change, and in parallel, more ROMs are produced (Figs. 1C and 4D). Thus, the metabolic patterns (direction, timing, and number) all are correlated strongly with the physiological release of ROMs. In the present studies FMLP was added uniformly to the cells to induce chemokinesis, thus FMLP concentration gradients or specific sites of receptor ligation cannot explain local ROM release. Furthermore, chemotactic factor receptors and their attendant G proteins are distributed uniformly in cell membranes (30). Thus, the local production of ROMs is not likely to be explained by the distribution of FMLP or its receptor.

Immunologic effector functions such as the destruction of target cells can be mediated by several mechanisms including the discharge of granule contents and the production of ROMs. The activation of natural killer cells is a particularly good example of the former, whereas neutrophil activation exemplifies the latter. Natural killer cells, as well as other types of immune cells, can discharge their contents vectorially toward a target (31). Previous findings suggested that ROMs are not released uniformly about the perimeter of neutrophils (22). Moreover, neutrophils with multiple attached targets have been shown to expose each target cell to ROMs sequentially, not simultaneously (32). Thus, ROMs may be released vectorially from neutrophils, but a mechanism to account for this has remained elusive. One potential mechanism that could account for local ROM release is the local availability of the NADPH oxidase’s substrate NADPH. Because metabolism is distributed inhomogeneously throughout a cell in the form of chemical waves (12–14), these waves might explain the focused and periodic release of ROMs. In polarized neutrophils traveling NAD(P)H waves have a specific direction, and consequently ROM production exhibits directional properties as well, being primarily released from the lamellipodium (Figs. 1 and 2). In other words, metabolic self-organization allows a polarized neutrophil to take aim at a specific site, thereby minimizing collateral damage to other nearby tissues while delivering high concentrations of toxic ROMs in the direction of its quarry. Traveling NAD(P)H waves also may account for periodic ROM production, because there are periods of high NAD(P)H concentration separated by longer periods of low NAD(P)H concentration. Periodic and focused ROM release may account for the periodic release of cytolytic markers (i.e., membrane disruption) from individual tumor cells during neutrophil-mediated cytotoxicity (33). NAD(P)H waves compress the delivery of electrons to NADPH oxidase into short pulses at a high concentration. Target sites are not exposed to slowly changing ROM levels but rather to periodic high concentrations; thus, the formation of dissipative NAD(P)H patterns may provide an important biological advantage in target destruction.

Although our studies have focused on metabolic self-organization and local ROM release, the implications of the concepts described above likely extend much further. For example, superoxide anions also may act as a paracrine signaling agent to affect the behavior of other nearby cells and tissues (34). NAD(P)H waves may contribute to the activities of additional enzymes. For example, NADPH is also a substrate for nitric-oxide synthase. Thus, NO synthesis in its many physiological settings, from its role in the formation of reactive

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nitrogen metabolites in leukocyte effector function to its role in vasodilation, may be controlled similarly. In addition to traveling throughout a single cell, preliminary studies in this laboratory have suggested that NAD(P)H waves can propagate among cells within confluent monolayers. Other high-speed imaging studies in this laboratory suggest that calcium signaling, another type of intracellular excitable matrix, is equally rich in temporal and spatial information. Further studies of dissipative structures within cells and tissues are likely to impact our understanding of the biochemistry of living cells.

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